*Atherosclerosis*, 31 (1978) 259—271


# COPPER DEFICIENCY AND CHOLESTEROL METABOLISM IN THE RAT

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(Received 6 March, 1978)
(Revised, received 31 July, 1978)
(Accepted 11 August, 1978)

## Summary

The influence of copper deficiency on both $^3H$ incorporation into plasma and liver lipids following [$^3H$]mevalonate injection, and the excretion of biliary steroids from cannulated bile ducts was examined in the rat in two separate experiments. Copper-deficient rats exhibited a significant hypercholesterolemia (129% increase, $P < 0.001$) at 181 days. Four hours after [$^3H$]mevalonate injection, $^3H$ incorporation into plasma free and esterified cholesterol was elevated 162% ($P < 0.005$) and 80% ($P < 0.025$), respectively, and $^3H$ incorporation into liver total lipids and cholesterol esters was depressed 18% ($P < 0.04$) and 43% ($P < 0.005$), respectively, in copper-deficient rats. Hepatic copper concentration of deficient rats showed a significant negative correlation with $^3H$ incorporation into plasma cholesterol ($r = -0.83$, $P < 0.02$) and cholesterol ester ($r = -0.70$, $P < 0.08$). Total hepatic cholesterol remained unchanged by deficiency despite the significant increase in total plasma cholesterol burden of copper deficient rats (82%, $P < 0.001$). Copper deficiency failed to impair biliary steroid excretion. The 56% ($P < 0.001$) increase in bile acid excretion in the first 8 hours was paralleled by the 62% ($P < 0.005$) increase in plasma cholesterol concentration of deficient rats.

The observations suggest a more rapid synthesis and clearance of cholesterol to the plasma pool, with this cholesterol being unavailable for excretion as biliary steroids, in copper-deficient rats.

Key words: *Atherosclerosis — Bile acid excretion — Cholesterol synthesis and clearance — Dietary copper deficiency — Plasma cholesterol*

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## Introduction

An increase in plasma [1], or serum [2], cholesterol concentration is one of the principal indications of ischemic (coronary) heart disease risk, the leading cause of death in the United States [3]. Accumulations of large amounts of lipids, principally cholesterol esters, in the atheromatous plaque often are associated with occlusive coronary disease [4]. Recent reports have suggested a role for copper in the metabolism of cholesterol, and dietary copper deficiency has produced hypercholesterolemia in rats [5—7]. It has been hypothesized that a relative or absolute deficiency of copper is a major factor in the etiology of coronary heart disease because of an association with many apparently dissimilar observations on the epidemiology of coronary heart disease and cholesterol metabolism [8].

Copper is essential for hematopoiesis [9], the lysyl oxidase cross-linking of connective proteins [10], and in several mixed-function oxygenases and cytochromes [11—13]. The production of hypercholesterolemia in copper-deficient rats suggests a new biochemical role for copper. Moreover, while previous reports of copper deficiency-induced hypercholesterolemia have documented changes in plasma cholesterol concentrations, they provide little information on the mechanism by which copper affects cholesterol metabolism [5—7]. It has been suggested that the hypercholesterolemia caused by copper deficiency is a consequence of reduced bile acid synthesis and excretion [6,7], an enhanced clearance of cholesterol from the liver to the plasma pool [6,7] or to a reduction in extra-hepatic cell utilization of circulating cholesterol [7]. However, no changes of in vitro cholesterol synthesis have been observed in livers from copper-deficient rats [6].

This report describes the influence of copper deficiency on both $^{3}H$ incorporation into plasma and liver lipid following [$^{3}H$]mevalonate injection, and the synthesis and excretion of biliary steroids.

## Materials and Methods

In two separate experiments weanling male Sprague-Dawley rats, weighing approximately 43 g each, were obtained from Bio Lab Corp., White Bear Lake, MN *. Rats were assigned to groups, matched by mean weight to within 0.4 g. The rats were fed a diet based on 51% sucrose, 20% egg white, 9.5% coconut oil and 9.5% cottonseed oil. The diet, which is free of cholesterol, contains all essential nutrients except copper. The diet is based on the recommendations of the National Academy of Sciences Committee on Animal Nutrition [14], and has been described in detail [7]. Experimental animals (copper-deficient) were fed the basal diet which contained 0.57 μg Cu/g diet; control animals were pair-fed [7] basal diet supplemented to 5.0 μg Cu/g diet, by the addition of finely ground reagent grade $CuSO_4 \cdot 5H_2O$. Diet analysis was accomplished by wet ashing with concentrated nitric and sulfuric acids followed by complete diges-

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tion with 50% hydrogen peroxide [15]. Copper was determined by flame atomic absorption spectrophotometry (Perkin Elmer Model 503, Norwalk, CT). Analysis of diets by this procedure has shown a 98% recovery for copper, and dietary copper to be within 5% of the desired concentration in both experimental and control diets.

In separate experiments where copper-deficient and control rats were fed the same experimental diets *ad libitum*, a very similar hypercholesterolemia was observed with copper-deficient animals. For example, at 70 days, *ad libitum* fed copper-deficient rats and *ad libitum* fed control rats showed a mean ± SEM plasma cholesterol concentration of 149.9 ± 20.6 mg/dl and 75.2 ± 3.8 mg/dl, respectively. The 99.4% ($n = 23$, $P < 0.001$) increase in plasma cholesterol concentration associated with copper deficiency at 70 days illustrates that the observed hypercholesterolemia is a consequence of dietary copper deficiency and is not a result of the restricted food intakes associated with pair feeding. Similar results have been reported with rats fed copper-deficient and control diets *ad libitum* [6].

*Experiment 1*

In a preliminary study control rats (approximately 400 g each) were injected subcutaneously with a total of 5 μCi [$^3$H]mevalonate (neutral bicarbonate buffer). Plasma was obtained at 2 and 4 h after injection and plasma lipids extracted (2 : 1 chloroform/methanol). Total plasma lipids were counted for $^3$H by liquid scintillation. The percent dose of $^3$H incorporated into total plasma lipids, per ml of plasma, was $1.6 \times 10^{-2}$ and $3.1 \times 10^{-2}$ at 2 and 4 h, respectively. Based on this preliminary study a 4-h period was chosen for $^3$H incorporation measurements.

Rats were assigned to two groups of 7 animals each, and each member of the experimental group was paired by weight with a member of the control group with a mean absolute difference between pairs of 0.9 g. Rats were fed the appropriate diet for 181 days and weighed weekly. At 181 days, the 7 pairs of rats were fasted for 24 h and injected, subcutaneously, at a dose of 3.0 μCi/100 g body weight, with freshly prepared [$^3$H]mevalonate (DL-[5-$^3$H]mevalonate, 5.0 Ci/mmol; New England Nuclear) in neutral 0.2 M sodium bicarbonate buffer. Four hours after injection, the rats were anesthetized with ether and killed by exsanguination (cardiac puncture). Blood was collected and centrifuged in heparin-treated tubes. Plasma samples were removed from packed cells and placed in metal-free polypropylene tubes (Falcon Tubes, Becton Dickinson and Co., Parsippany, NJ). Livers were excised, promptly frozen in acetone/dry ice, and stored at —90° C.

The lipids in 1.0 ml of plasma, and approximately 1.0 g samples of liver, were extracted with 30.0 ml of chloroform : methanol (2 : 1, v/v) and refluxed gently at 60° C for 1 h. Filtered extracts were washed with $\frac{1}{5}$ the extract volume of 0.1 M sodium chloride, shaken, and the phases allowed to separate at room temperature. The chloroform phase was removed and evaporated using a rotary evaporator. Lipids were immediately redissolved in chloroform and stored at 4° C. Plasma and liver lipids were redissolved in 2.0 ml and 5.0 ml of chloroform, respectively. Samples of both plasma and liver lipid extracts from each rat, 0.5 ml each, were pipetted into liquid scintillation vials, and the chlo-

roform purged with nitrogen. $^{3}H$ incorporation into total lipids was determined by liquid scintillation (Nuclear Chicago) in 10.0 ml of Aquasol-2 (New England Nuclear). The major lipid fractions of plasma and liver extracts were also separated by thin layer chromatography on silica gel H (20 × 20 cm, 250 μm, Applied Science Laboratories, College Station, PA), freshly activated at 110°C for 1 h. Lipid extracts, 200—500 μl, were applied as a band 2.5 cm wide and lines were ruled between samples to prevent migration. Standard samples of pure cholesterol and cholesteryl oleate (Applied Science Laboratories) were also chromatographed. Chromatograms were developed using a hexane : diethyl ether : acetic acid (80 : 20 : 2, v/v) solvent system that separates the major lipid classes [16]. The solvent front was allowed to rise to within 3 cm of the top of the plate. Chromatograms were air-dried, and lipid zones located with iodine vapor and by reference to the standard samples. Silica gel regions corresponding to cholesterol and cholesteryl esters were scraped into liquid scintillation vials. Unstained areas of similar size to the lipid zones were removed and counted as blanks. All samples were corrected for quenching by use of quenched standards.

Plasma cholesterol was determined fluorometrically by the method of Carpenter et al. [17]; liver cholesterol was determined in a similar manner following extraction with 2 : 1 chloroform : methanol. The copper content of plasma and liver was determined by flame atomic absorption spectrophotometry using plasma samples diluted with distilled demineralized water, and liver samples, approximately 1.0 g each, digested as described for diet analysis (*vide supra*) after lyophilization.

*Experiment 2*

Rats were assigned to two groups of 10 animals; each member of the experimental group (copper-deficient) was paired by weight with a member of the control group with a mean absolute difference of 0.3 g between pairs. Rats were fed appropriate diets for 36 days and weighed weekly. Commencing on day 36, pairs of rats were fasted for 12 h, anesthetized with ether, and the bile ducts cannulated. Bile ducts were ligated with silk and allowed to distend for a few minutes prior to cannulation. Polyethylene tubing (0.024 in. OD, PE10, Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ) was inserted through the bile duct wall, and secured by silk ligatures. Following bile duct cannulation, the polyethylene tubing was threaded subcutaneously and exited at the inter-scapular region. Cannulae were passed through 3/16 in. Tygon Tubing, securely sutured to rat skin, and threaded inside a restraint spring attached to the top of cage. Bile duct cannulation was performed according to a modification of the procedure of Kleinman et al. [18,19]. Cannulated rats were maintained in metabolic cages, fed the appropriate diets *ad libitum*, and provided with Ringer's solution, prepared with distilled demineralized water, to protect them against electrolyte loss. Bile samples were collected and measured in ice-chilled tubes during 0—8, 8—24, 24—48, and 48—72 h intervals. Pairs of rats, an experimental animal and the corresponding pair-fed control, were treated in this manner on subsequent days after day 36 until all 10 pairs of rats had been cannulated by day 63. Two control rats died during surgery, and two additional rats, one experimental and one control animal, died

during the 0—8 h bile collection period. All the remaining rats, 9 experimental and 7 control animals, had good flow for at least 24 h after the last collection period. Blood was obtained from each rat, at the time of cannulation, by tail vein bleeding into heparin-treated microhematocrit tubes, and promptly centrifuged. Plasma cholesterol was determined fluorometrically [17]. Biliary copper was determined by flame atomic absorption spectrophotometry using bile samples diluted with distilled, demineralized water.

Total biliary cholesterol was determined spectrophotometrically [20] using cholesterol esterase (EC. 1.1.3.6) and cholesterol oxidase (EC. 3.1.1.13) (Cholesterol Enzymatic; Boehringer Mannheim, Mannheim, FDR). Bile samples, 2.0 ml each, were hydrolyzed by autoclaving in 1.25 M KOH at 18 psi, 121°C, for 3 h. Cooled hydrolysates were extracted with two 50-ml portions of diethyl ether to remove non-saponifiable components, and the ether extracts backwashed. The ether extracts were discarded and the aqueous solution was acidified to pH 2 with 4 M hydrochloric acid. The acidified solution was extracted with three 50-ml portions of diethyl ether. Pooled ether extracts were evaporated with a rotary evaporator at 30°C, redissolved in absolute ethanol to a final volume of 5.0 ml, and stored at 4°C. Total biliary bile acids were determined enzymatically, using 3α-hydroxysteroid dehydrogenase (EC. 1.1.1.50, specific activity 0.86 units/mg; Worthington Biochemical Corporation, Freehold, NJ), by a modification of the procedure of Turnberg and Anthony-Mote [21]. Assay solutions (1.5 ml final volume) contained 0.85 ml 0.1 M sodium pyrophosphate pH 10.8, 0.5 ml 0.005 M $NAD^+$, 0.1 ml of a 10 mg/ml solution of 3α-hydroxysteroid dehydrogenase, and 0.05 ml bile acid extract in ethanol. Blanks were run in a similar manner except that 0.05 ml of ethanol was substituted for the ethanolic bile acid extract. Assay samples were incubated at 26°C for 60 min, and absorbance was read at 340 nm with a Cary 118 recording spectrophotometer. Bile acid concentration in the assay mixture was calculated according to the following equation:

$$\mu\text{moles } 3\alpha\text{-hydroxysteroid} = \frac{\Delta A \times 1.5}{6.22}$$

where $\Delta A$ = increase in absorption at 340 nm, and $6.22 \times 10^6$ = molar extinction coefficient of NADH at 340 nm.

Analysis of samples of pure cholic and chenodeoxycholic acids (Supelco Inc., Bellefonte, PA) by this method has shown results to be within 3.7% and 3.5%, respectively, of the theoretical values.

All experimental results were analyzed by Student's *t*-test and linear regression [22].

## Results

*Experiment 1*

The growth of copper-deficient rats was depressed by 20% at 181 days in comparison to pair-fed control animals (Fig. 1). At 181 days, copper-deficient rats exhibited a 129% ($P < 0.001$) (Table 1) increase in the plasma cholesterol concentration, with no overlap between deficient and pair-fed control rats. Liver cholesterol concentration and total liver cholesterol were not significantly

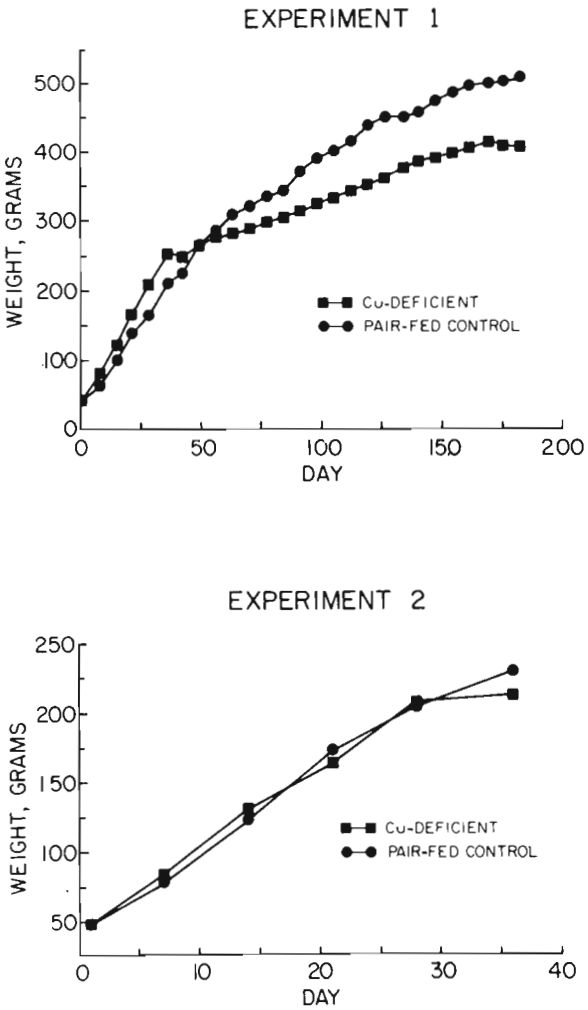


Fig. 1. Growth of copper-deficient and pair-fed control rats.

different (Table 1). Plasma and liver copper concentrations were reduced by 95% ($P < 0.001$) and 74% ($P < 0.001$), respectively, in copper-deficient animals (Table 1).

The percent dose incorporation of $^3H$, 4 h after [$^3H$]mevalonate injection, into plasma total lipids, plasma cholesteryl esters, and plasma cholesterol was

TABLE 1

EXPERIMENT 1: PLASMA AND LIVER CHOLESTEROL, AND COPPER CONCENTRATIONS IN COPPER-DEFICIENT AND PAIR-FED CONTROL RATS AT 181 DAYS, $\bar{x}$ ± SEM

| | Plasma Cu (μg/ml) | Liver Cu (μg/g) [a] | Liver cholesterol (mg/g) [b] | Total liver cholesterol (mg) | Plasma cholesterol (mg/dl) |
|---|---|---|---|---|---|
| Copper-deficient rats | 0.08 ± 0.03 | 4.29 ± 0.62 | 2.44 ± 0.16 | 27.9 ± 2.5 | 109.6 ± 7.9 |
| | $P < 0.001$ | $P < 0.001$ | N.S. | N.S. | $P < 0.001$ |
| Control rats (pair-fed) | 1.58 ± 0.16 | 16.46 ± 0.47 | 2.78 ± 0.11 | 28.8 ± 1.8 | 47.9 ± 4.8 |

[a] Dry weight.
[b] Wet weight.

elevated 100% ($P < 0.01$), 80% ($P < 0.025$) and 162% ($P < 0.005$), respectively, in copper-deficient rats (Fig. 2). During this same time period, $^3H$ incorporation into liver total lipids and liver cholesteryl esters was reduced by 18% ($P < 0.04$) and 43% ($P < 0.005$), respectively, in copper-deficient rats in comparison to pair-fed control animals (Fig. 2). However, no difference in $^3H$ incorporated into liver cholesterol was observed between copper-deficient rats and pair-fed control animals (Fig. 2). Liver copper concentration, μg/g, in copper-deficient rats showed a significant negative correlation with $^3H$ incorporation into plasma total lipids ($r = -0.73$, $P < 0.05$), plasma cholesteryl esters ($r = -0.70$, $P < 0.08$) and plasma cholesterol ($r = -0.83$, $P < 0.02$).

*Experiment 2*

At 36 days, there were no significant differences in the weights of copper-deficient and pair-fed control rats (Fig. 1). Copper-deficient rats exhibited a 62% ($P < 0.005$) increase in plasma cholesterol concentration in comparison to pair-fed control animals, measured at the time of bile duct cannulation. Biliary copper analysis was used as an index of copper status, and copper-deficient rats showed a >98% ($P < 0.001$) reduction in biliary copper excretion during the first 8-h period. Copper was undetectable in later bile samples from deficient rats and remained essentially unchanged throughout the experiment (Table 2). The increase in plasma cholesterol concentration in copper-deficient rats, measured at the time of cannulae insertion, was closely paralleled by the increase in total bile acid excretion during the first 8-h period, 62% ($P < 0.005$) and 56% ($P < 0.001$), respectively (Table 2). During the 8—24-h collection period, copper-deficient animals continued to excrete 85% ($P < 0.005$) more bile acids than pair-fed control animals (Table 2). By the 24—48-h period, this difference had diminished to 23% (NS), and bile acid excretion was essentially

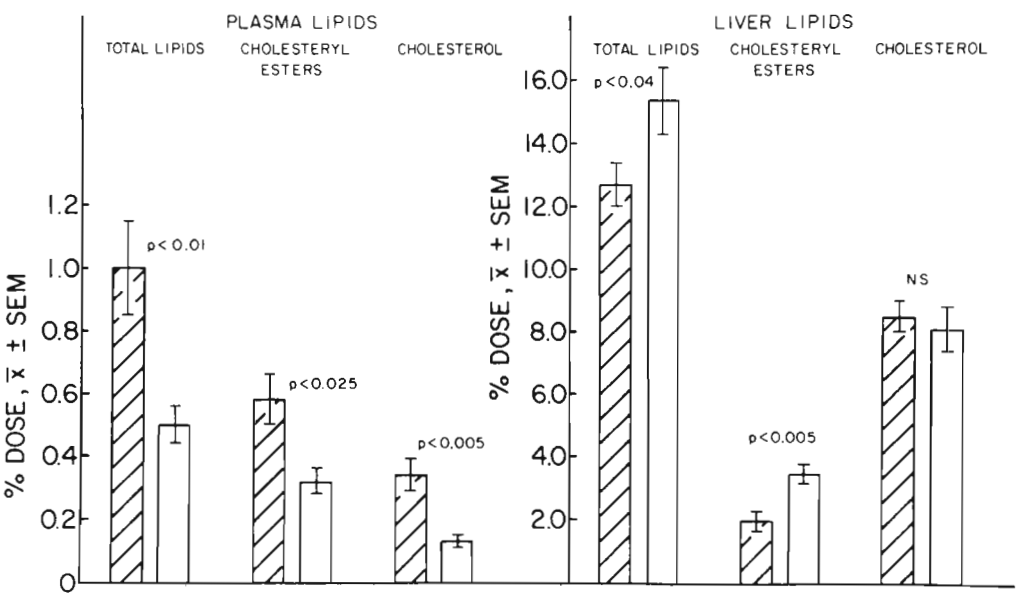


Fig. 2. Experiment 1: Percentage of the dose of [$^3H$]mevalonate appearing in plasma and liver lipids 4 h after [$^3H$]mevalonate injection. Results are expressed as percentage of total administered dose of [$^3H$]-mevalonate appearing in the total plasma lipid fractions, and percentage of administered dose appearing in total liver lipid fractions per 100 g body weight. ▨ Copper-deficient rats; □ pair-fed control rats.

TABLE 2

EXPERIMENT 2: BILIARY STEROID AND COPPER EXCRETION, AND PLASMA CHOLESTEROL CONCENTRATION IN COPPER-DEFICIENT AND PAIR-FED CONTROL RATS WITH BILIARY CANNULAE, $\bar{x} \pm$ SEM

| | Time period (h) | Copper-deficient rats | | Control rats (pair-fed) |
|---|---|---|---|---|
| Bile volume (ml) | 0—8 | 6.16 ± 0.35 | $P < 0.005$ | 4.35 ± 0.16 |
| | 8—24 | 8.76 ± 0.51 | ($P < 0.09$) | 6.72 ± 0.87 |
| | 24—48 | 11.07 ± 0.62 | | 9.91 ± 0.31 |
| | 48—72 | 10.33 ± 0.57 | | 9.83 ± 0.11 |
| Biliary Cu (μg/ml) | 0—8 | 0.03 ± 0.01 | $P < 0.001$ | 1.64 ± 0.48 |
| | 8—24 | < 0.01 | $P < 0.001$ | 1.54 ± 0.16 |
| | 24—48 | < 0.01 | $P < 0.001$ | 0.91 ± 0.16 |
| | 48—72 | < 0.01 | $P < 0.001$ | 0.83 ± 0.20 |
| Biliary bile acids (μmoles 3α-hydroxysteroid/ml) | 0—8 | 15.23 ± 0.94 | | 13.68 ± 0.96 |
| | 8—24 | 7.95 ± 0.56 | | 6.21 ± 0.89 |
| | 24—48 | 8.36 ± 0.56 | | 7.54 ± 0.39 |
| | 48—72 | 11.28 ± 0.52 | | 11.19 ± 0.55 |
| Biliary cholesterol (mg/dl) | 0—8 | 13.24 ± 0.79 | | 13.86 ± 1.74 |
| | 8—24 | 12.76 ± 1.09 | $P < 0.01$ | 7.73 ± 1.03 |
| | 24—48 | 16.88 ± 0.59 | | 16.88 ± 1.50 |
| | 48—72 | 17.36 ± 0.83 | | 17.48 ± 1.40 |
| Total biliary bile acids (μmoles 3α-hydroxysteroid) | 0—8 | 93.66 ± 6.76 | $P < 0.001$ | 59.91 ± 2.27 |
| | 8—24 | 70.87 ± 6.72 | $P < 0.005$ | 38.34 ± 3.03 |
| | 24—48 | 91.57 ± 7.16 | | 74.73 ± 4.67 |
| | 48—72 | 116.48 ± 7.98 | | 110.05 ± 5.82 |
| Total biliary cholesterol (mg) | 0—8 | 0.80 ± 0.06 | ($P < 0.09$) | 0.60 ± 0.07 |
| | 8—24 | 1.08 ± 0.15 | $P < 0.025$ | 0.56 ± 0.06 |
| | 24—48 | 1.86 ± 0.08 | | 1.67 ± 0.14 |
| | 48—72 | 1.88 ± 0.13 | | 1.72 ± 0.13 |
| Plasma cholesterol [a] (mg/dl) | | 100.0 ± 8.2 | $P < 0.005$ | 61.7 ± 4.0 |

[a] Measured at the time of cannula insertion.

the same in copper-deficient and pair-fed control animals by the 48—72-h period (Table 2). A modest increase in bile acid production and excretion of 24% (NS) was observed in copper-deficient rats between the initial and final bile collection periods, in response to bile diversion. Control animals, on the other hand, increased bile acid production by 85% ($P < 0.001$) during this same period.

In both copper-deficient and control rats, bile acid production and excretion decreased during the 8—24-h collection period, 32% and 54%, respectively, which was followed by a steady increase during the remainder of the experiment. Total biliary cholesterol excretion in both groups of rats was small in comparison to bile acid excretion. Biliary cholesterol comprised 2.2—5.0 mole% of total biliary steroids throughout the course of the experiment, with no significant differences in cholesterol mole% between copper-deficient and control rats. The differences in total biliary cholesterol excretion between

copper-deficient and control rats paralleled changes in bile acid excretion. During the 0—8-h and 8—24-h collection periods, copper-deficient rats excreted 33% (NS) and 93% ($P < 0.025$), respectively, more cholesterol than control animals (Table 2). Total biliary cholesterol excretion was similar in both groups of animals during the subsequent 24—48 and 48—70-h collection periods. Bile acid concentration, μmoles/ml, was increased a modest 11—28% in copper-deficient rats (Table 2) throughout the experiment; however, this increase was not statistically significant. The concentration of cholesterol in bile was essentially unchanged by copper deficiency except during the 8—24-h collection period when copper-deficient animals exhibited a 65% ($P < 0.01$) increase in biliary cholesterol concentration (Table 2). Bile flow was increased by copper deficiency during the 0—8-h and 8—24-h collection periods, 42% ($P < 0.005$) and 30% (NS), respectively. However, in response to bile diversion, bile volumes were similar by the 48—72-h collection period in both groups of animals.

## Discussion

Mevalonic acid is an obligatory intermediate in the biosynthesis of cholesterol [23], and the formation of mevalonate from 3-hydroxy-3-methyl glutarate is the principal control point in cholesterol biosynthesis [23]. Despite the fact that the synthesis of endogenous mevalonate and cholesterol occurs predominantly in the mammalian liver [24], studies in the rat have demonstrated that the kidney metabolizes a significant portion of the mevalonate administered by injection [24]. The products of renal mevalonate metabolism are squalene and lanosterol; however, the kidney shows an insignificant ability to convert these precursors to cholesterol [24]. The lanosterol and squalene produced by renal tissue are metabolized to cholesterol in the liver [24]. Consequently, $^3H$ incorporation into plasma and liver cholesterol pools, following [$^3H$]mevalonate injection, provides a measure of the net influx to, and efflux from, the plasma and liver cholesterol pools during a given interval. Changes in $^3H$ incorporation in liver and plasma cholesterol pools are dependent upon cholesterol synthesis, clearance to the plasma, bile acid synthesis (cholesterol degradation) and biliary excretion, and on the uptake of cholesterol by extrahepatic tissue. The amount of mevalonate injected in these studies, 0.08 μg L-mevalonate (0.16 μg DL-mevalonate) per 100 g body weight represents 5.6% of the estimated pool of 1.43 μg L-mevalonate/100 g body weight [24], an amount unlikely to perturb cholesterogenesis significantly.

The marked increase in $^3H$ incorporation into both plasma cholesterol (80%, $P < 0.025$) and plasma cholesteryl esters (162%, $P < 0.005$) is consonant with the increase in plasma cholesterol concentration (129%, $P < 0.001$) observed in copper deficiency (Table 1), and suggests a role for copper in cholesterol metabolism. The increased $^3H$ incorporation into both free and esterified plasma cholesterol observed 4 h after [$^3H$]mevalonate injection, was paralleled by a reduction in $^3H$ incorporation into total liver lipids in copper-deficient rats (Fig. 2). The reduction in $^3H$ incorporation into liver lipids was limited to an effect on cholesteryl esters, and $^3H$ incorporation into free cholesterol in the liver was essentially unchanged in both groups of rats (Fig. 2). The similarity

of $^{3}H$ incorporation into hepatic free cholesterol is most probably a result of the time period chosen. The incorporation of $^{3}H$ into both free and esterified plasma cholesterol in copper-deficient rats was increased by 162% ($P < 0.005$) and 80% ($P < 0.025$), respectively (Fig. 2), 4 h after [$^{3}H$]mevalonate injection, and indicates a more rapid synthesis and clearance of cholesterol to the plasma pool in copper deficiency. Consequently, by 4 h $^{3}H$ incorporation into hepatic free cholesterol may have peaked and be decreasing in copper-deficient rats while it is still increasing in the control animals, and hence appears to be of approximately equal magnitude in both groups of animals (Fig. 2). It would, perhaps, seem possible that copper deficiency may increase the rate of conversion of mevalonate to lanosterol and squalene precursors in renal tissue and, hence, enhance the appearance of $^{3}H$ label in the livers of copper-deficient rats, where the squalene and lanosterol are metabolized to cholesterol. Such an effect would accelerate the appearance of $^{3}H$ in the plasma cholesterol pool of copper-deficient rats. However, this explanation is unlikely since it fails to explain the hypercholesterolemia observed in copper-deficient rats where exogenous mevalonate has not been injected and, hence, renal mevalonate metabolism is insignificant.

Lei has reported no significant differences in the in vitro synthesis of cholesterol from [$^{14}C$]acetate in the livers of copper-deficient and copper-adequate rats [6]. However, if we assume a plasma volume of 40.4 ml/kg for the rat [25], the mean total plasma cholesterol content of copper-deficient and pair-fed control rats is 17.1 mg and 9.4 mg, respectively. This 7.7 mg (82%, $P < 0.001$) increase in total plasma cholesterol burden associated with copper deficiency was not matched by changes in total hepatic cholesterol in copper-deficient rats, despite the small but insignificant reduction in hepatic cholesterol concentration associated with copper deficiency (Table 1). These results are consonant with the $^{3}H$ incorporation data and suggest that newly synthesized cholesterol is cleared to the plasma pool more rapidly in copper deficiency.

It has been suggested that the hypercholesterolemia observed in copper deficiency is a consequence of reduced bile acid synthesis and biliary excretion [6,7]. The mixed-function oxygenase (monooxygenase) hydroxylations of the steroid nucleus [26,27] in bile acid synthesis show some similarities to other copper requiring monooxygenases [12]. The data in Table 2 show that copper deficiency does not impair synthesis or excretion of bile acids. If it is assumed that the first 8-h collection period approximates the in vivo production and secretion of bile acids, the increase in bile acid excretion (56%, $P < 0.001$) in copper-deficient rats during this period closely matches the increase in plasma cholesterol excretion (62%, $P < 0.005$) associated with copper deficiency (Table 2). Moreover, the maximum production and excretion of bile acids during the 48—74-h collection period, in response to the biliary diversion, was very similar in both copper-deficient and control animals (Table 2). Data on biliary cholesterol excretion parallel the changes in bile acid excretion (Table 2). Biliary cholesterol excretion constitutes only 2.2—5.0 mole% of biliary steroids and no significant differences in cholesterol mole% were observed either with time, or between copper-deficient or control rats. The >98% ($P < 0.001$) reduction in biliary copper excretion, observed throughout the

course of the experiment, in copper-deficient rats is in agreement with the observations of Owen and Hazelrig [28], and probably reflects copper conservation in response to deficiency.

Earlier reports have noted no significant differences in hepatic cholesterol concentration between copper-deficient and control rats despite significant increases in plasma cholesterol associated with copper deficiency [6,7]. Furthermore, at 181 days (Experiment 1, Table 1) the total hepatic cholesterol content of copper-deficient and control rats, 27.9 and 28.8 mg, respectively, are remarkably similar despite the 129% increase in plasma cholesterol concentration observed in copper-deficient animals (Table 1). The inability of biliary steroid excretion to compensate for hypercholesterolemia in copper deficiency is, however, not attributable to a role for copper in bile acid synthesis and excretion since maximum biliary steroid production, in response to bile diversion, is very similar in both copper-deficient and control animals by 48—72 h (Table 2). The failure of biliary steroid excretion to counteract the copper deficiency-induced hypercholesterolemia may reflect the more rapid clearance of newly synthesized cholesterol to the plasma pool in copper-deficient rats. Cholesterol in plasma is unavailable for conversion to bile acids, with subsequent biliary elimination, which represents the only quantitatively significant pathway for cholesterol excretion [29].

The results of this study indicate that a more rapid synthesis and clearance of cholesterol to the plasma pool is associated with copper deficiency. The enhanced clearance of cholesterol to plasma renders this cholesterol pool unavailable for catabolism and excretion as bile acids. Cholesterol transport in the plasma is dependent upon plasma lipoproteins [30]. The efflux of cholesterol from liver, and its transport to peripheral tissues, is effected by plasma low density lipoproteins (LDL) [30,31]. The LDL receptor-mediated uptake of cholesterol ensures that these peripheral (extra-hepatic) tissues utilize cholesterol of hepatic origin for their metabolic needs [30,31]. More recent evidence suggests that the high density lipoproteins (HDL) transport cholesterol from extra-hepatic tissue for redelivery to LDL, or to liver for cholesterol excretion as bile acids [30,32—34]. The influence of copper deficiency on cholesterol clearance to the plasma pool suggests a role for copper in lipoprotein metabolism.

The marked increase in cholesterol clearance to the plasma that accompanies copper deficiency may be of importance in the etiology of coronary (ischemic) heart disease. The concentration of cholesterol in plasma is predictive of risk of coronary heart disease [1], and recent analysis of both institutional [35—37] and self chosen diets [38,39] has shown many to contain considerably less than the suggested 2 mg daily requirement of copper [40,41]. Although the observations reported in these studies were obtained using a cholesterol-free diet, these observations may also apply to diets commonly consumed in the United States which are typically high in cholesterol. Both endogenously synthesized cholesterol and dietary cholesterol, transported to the liver by chylomicrons remnants [42,43] contribute to the hepatic pool. Consequently, the entry of cholesterol into the plasma pool may also be further enhanced when diets are both high in cholesterol and low in copper content.

Finally, these observations, although limited to the rat, suggest that adequate

and well defined dietary copper concentrations are necessary in studies on cholesterol metabolism in both laboratory animals and people, and that dietary copper should be evaluated in epidemiologic studies.

## References


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